

DEVELOPMENT OF A BIOASSAY FOR EVALUATING TICK (ACARI: IXODIDAE) ATTRACTANTS IN THE FIELD

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Abstract.—A method for testing tick attractants under field conditions is described. The uneven distribution of host-seeking ticks, even within habitat types, can compromise evaluations of experimental baits and necessitate extensive testing. This problem can be remedied by releasing known numbers of ticks at predetermined locations and distances from an experimental bait. Lone star tick, *Amblyomma americanum* (L.), nymphs were released directly downwind of dry ice baits on 2.25 × 2.25 m cotton sheets that had been placed on the ground. Each sheet was aligned with the direction of the wind and edged with a masking tape barrier. Best results in discriminating between dry ice baits and untreated controls were with nymphs released 1.5 and 2 m from the bait and recaptured after 1 h in a zone ≤1 m from the bait. The success of this method depends on the wind not stopping for prolonged periods and not radically changing direction.

Key Words: lone star tick, *Amblyomma americanum*, carbon dioxide, dry ice

The efficacy of any tick attractant system is measured by its performance under field conditions. Using natural populations to evaluate a tick attractant in the field can involve extensive replication if the tick population sampled is not dense. The tendency for clumped distributions among host-seeking ticks, even within habitat types, exacerbates the likelihood that baits will be placed in unproductive locations. For instance, Newhouse (1983) found that along park trails in Georgia adult American dog ticks, *Dermacentor variabilis* (Say), were nonrandomly distributed in clusters that changed annually. Solberg et al. (2003) also found extreme clumping of blacklegged ticks, *Ixodes scapularis* Say, in study plots in Maryland. If few or no responsive ticks are within the effective range of an experimental bait, little is learned from the trial.

An alternative to using natural populations is to seed the area around an experimental bait with marked host-seeking ticks, thereby assuring that potentially responsive ticks will be exposed to the attractant (Falco and Fish 1991).

Carbon dioxide (CO₂) has been used as an attractant for ixodid and argasid ticks for decades (Garcia 1965, Semtner et al. 1975, Falco and Fish 1991). Holscher et al. (1980) elucidated electrophysiological responses of three species of ticks to CO₂. Along with flagging and dragging, dry ice baiting has been a method of choice for collecting many host-seeking ticks and monitoring their populations (Gladney 1978, Koch 1987, Koch and McNew 1981, Solberg et al. 1992). Highly mobile tick species travel considerable distances to CO₂ sources. Lone star ticks, *Amblyomma americanum*

(L), have been reported to be attracted to CO₂ sources from as far as 21 m (Wilson et al. 1972). Although long duration dry ice traps that operate for days have been developed (Gray 1985), most dry ice baiting is done with small blocks that last for only for 1–2 h (Koch 1987). A drawback in distributing dry ice to numerous sample sites in the field is carrying several kilograms on transects or to remote plots in tick habitat and placing the baits before sublimation significantly diminishes the last pieces of dry ice.

The purpose of this study was to develop a method for testing attractants in the field that used a modest number of released ticks and provided statistically discriminatory data about the efficacy of the bait. Three variations of the basic premise of releasing ticks directly downwind of a bait in the field were tested sequentially in 2003 and 2004 in an attempt to maximize the numbers of attracted ticks recaptured. We report the basic features and requirements of a tick attractant field bioassay.

MATERIALS AND METHODS

We conducted one set of trials to demonstrate the variation encountered when using natural populations for tick attraction experiments. Three sets of trials were conducted using marked-released-recaptured ticks to define the basic elements of a method to reliably test an experimental tick attractant under field conditions.

Trials with natural tick populations.—To ascertain variation in nymphal densities at the geographic scale at which a tick attractant would operate, trials using dry ice baits were conducted on seven dates during the late spring and summer of 2004 in mesic forests at Fort A. P. Hill, Virginia and the Patuxent Wildlife Research Center, Laurel, Maryland. The predominant species and life stage present were *A. americanum* nymphs. To increase the likelihood that host-seeking ticks were in attractable range, bait locations were selected ≥ 2 h in advance of testing by flagging for host-seeking

ticks with 0.5 × 0.5 m crib cloth (a laminate of flannel and rubber). At locations where at least four nymphs were captured in five 30-sec bouts of flagging (each roughly linear) that covered in total a $\approx 15 \times 15$ m area, a marking flag was placed near the center of the flagged area. A 1 × 1 m piece of white nylon sail cloth was spread, as level as possible, on the substrate. A 500–550 g block of dry ice was placed on an inverted styrofoam cup (10 cm diam × 5 cm high × 7.5 cm diam) that had been positioned on the center of the cloth. Elevating the dry ice on the cup may aid in dispersal of the CO₂, which is heavier than air. As a control, an inverted cup without the dry ice was placed on a separate sheet at a location ≥ 75 m distant where ≥ 4 *A. americanum* nymphs were found by flagging. After 2 h, ticks on the upper and under sides of the cloths were collected on transparent tape and affixed in a notebook for subsequent identification and counting. A Bacharach CO₂ Analyzer, Model 2815 (New Kensington, PA) was used to obtain measurements of CO₂ in the air at ground level. Relative humidity (RH) and temperature on the upper surface each sheet were measured with a digital hygrothermometer (Model EA25, Extech Instruments, Waltham, MA). A total of 11 pairs of treatment and control sheets was tested.

Trials with marked-released-recaptured ticks.—The *A. americanum* nymphs used in the mark-release-recapture trials were from a laboratory colony at United States Department of Agriculture, Agricultural Research Service, Knipling-Bushland U. S. Livestock Insects Research Laboratory, Kerrville, Texas. The ticks were maintained at 24°C, $\approx 97\%$ RH and 16:8 h (L:D) until used in the trials.

Trials were conducted on large (2.25 × 2.25 m) white cotton sheets (45 × 45 thread mesh/cm). Each sheet was marked with three arcs having radii of 0.3, 1.0 and 2.0 m from one corner of the sheet, a pattern referred to hereafter as Configuration 1 (Fig. 1). A marking flag was placed into the

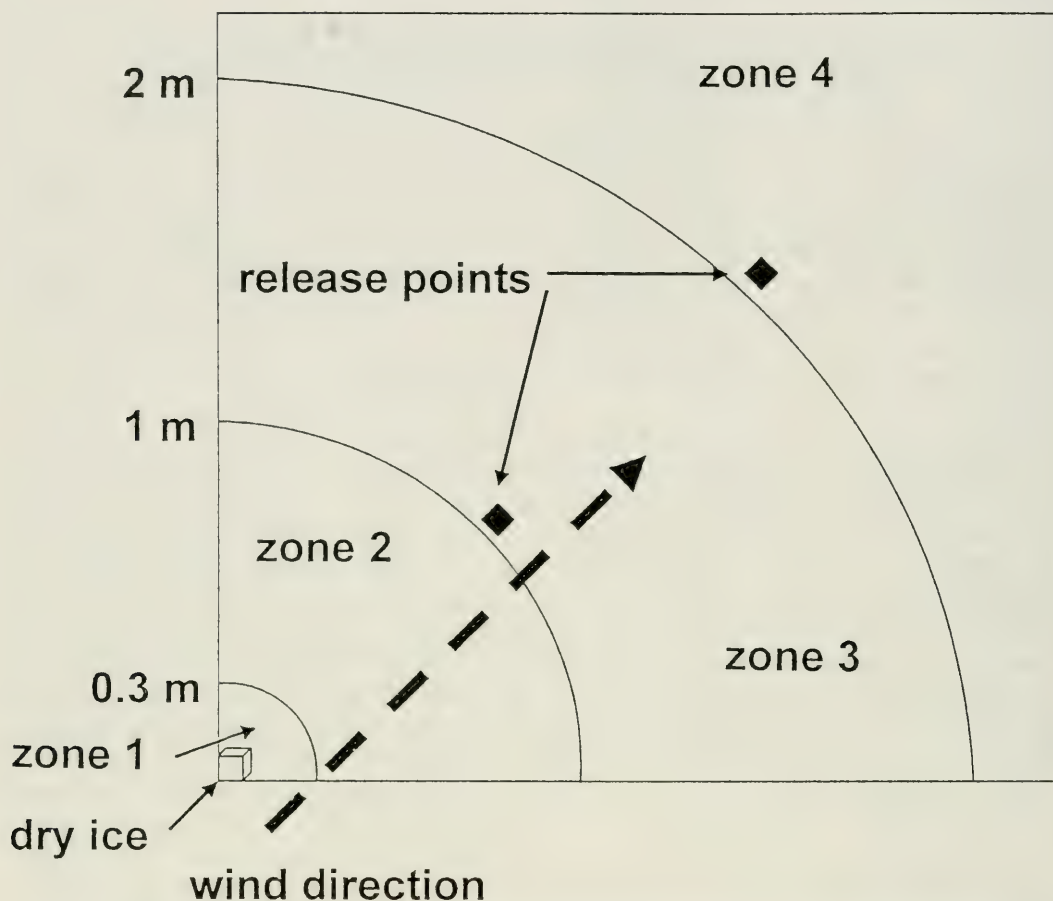


Fig. 1. Diagram showing 2.25×2.25 m sheet with bait location, recapture zones, tick release points for Configuration 1, and alignment with prevailing wind at start of attractant bioassay. The sheet was edged with masking tape to confine released ticks and exclude ticks from natural population.

ground to monitor wind direction. The corner of the sheet nearest the 0.3 m arc was placed at the base of the flag stake, and the sheet was adjusted so that the diagonal between the flag corner the opposite corner aligned with the direction of the wind, as indicated by the flag (Fig. 1). Masking tape (7.5 cm wide) was affixed to the edges of the sheets with the sticky side facing up and a 3–5 cm wide band of sticky surface exposed. At each corner, a flag stake was pushed through the tape into the ground to prevent billowing and folding over of the sheet. Two additional stakes were pushed through the tape on each upwind margin of the sheet, where there was a greater tenden-

cy for it to fold over in a strong breeze. As dictated by change in wind direction, the cloths were reoriented for each trial before securing them to the ground.

Configuration 1, Experiment 1. A plastic box (26.7 by 16.5 cm top, 21.0 by 13.3 cm bottom, 10.2 cm high) with a slit (15.6 by 0.6 cm long side, 8.5 by 0.6 cm short side) on each side, 2 cm above the bottom was placed in the upwind corner of the sheet, as close as possible to the masking tape edging. Dry ice (300–350 g) was placed on top of an inverted styrofoam cup inside the box. For the negative control, a box containing an inverted cup was placed on the upwind corner of the sheet. Ten nymphs marked

with colored dust (Switzer Brothers, Cleveland, OH) were released from a holding vial 1.01 m downwind from the corner of the sheet with the bait (dry ice or control), on the diagonal between the upwind and downwind corners (Fig. 1). Immediately thereafter a second group of 10 nymphs, marked with a different color dust, was released on the diagonal 2.01 m downwind from the bait corner. The placement of each color group at 1.01 or 2.01 m from the upwind corner was randomly determined for each sheet. One hour after the ticks were released, they were recaptured on pieces of 2.5-cm wide masking tape. The locations of their recapture were recorded as one of four zones referenced to the upwind corner: Zone 1 (<0.3 m), Zone 2 (0.3–1.0 m), Zone 3 (1.0–2.0 m), Zone 4 (>2.0 m). All tests were conducted at the same location, a narrow (5 m) grassy strip between a hedgerow of mature deciduous trees and a hay field. Dry ice and an untreated control were tested sequentially in random order (<1 h apart) on each of 4 d. The dry ice was weighed before and after each trial. Ambient temperature and humidity were recorded for each trial by placing a digital hygrometer on the sheet immediately before the start of each trial.

Configuration 1, Experiment 2. The sheets were similarly marked, taped, aligned and secured, as in Experiment 1. Either dry ice (300–350g) or no attractant (control) was placed in the upwind (bait) corner of each cloth. Each of two cloths was placed 100 m apart and ~3 m from the hedgerow. Unlike Experiment 1, no box and cup platform was used to protect and elevate the treatments. Once the treatments were in place, groups of 15–19 marked nymphs were released 1.01 and 2.01 m downwind from the bait, on the diagonal between the upwind and downwind corners of the sheet. Each of the two groups of ticks was marked with a different randomly assigned color. A dry ice sheet and untreated control sheet were tested in pairs, with a <10 min lag between the release of ticks

on the first and second sheet. One hour after the ticks were released they were recaptured and their locations recorded as in Experiment 1 (Fig. 1). Dry ice was weighed on site with a battery operated scale (Model CS5000, Ohaus Corporation, Pine Brook, NJ) immediately before and after each test. Temperature and RH were recorded on each sheet just before the start of each test. Two trials were conducted on each of 2 d.

Configuration 2. Trials were conducted on sheets of the same dimensions and fabric as in Configuration 1. However, the dry ice bait was located at the midpoint of one edge of the sheet, and the arcs for the 0.3, 1 and 2 m radii from the bait were drawn using the midpoint of the edge as origin (Fig. 2). A sheet was aligned so that the wind direction was at a right angle to the edge of the sheet causing the CO₂ plume to pass perpendicularly onto the sheet (Fig. 2). An inverted styrofoam cup was placed at the midpoint of the upwind edge of the sheet, as close as possible to the masking tape edging. A block of dry ice (308–328 g) was placed on top of the cup. Fifty marked *A. americanum* nymphs were released 1.5 m directly downwind of the cup. One hour later the ticks were collected on 2.5-cm wide masking tape, according to the zone in which they were found and returned to the laboratory for counting. Temperature, RH, wind speed (Kestrel 2000 Pocket Thermo Wind Meter, Nielsen Kellerman, Chester, PA) and background CO₂ readings were taken just before the dry ice was placed on the cup and just after the ticks were recaptured. Five trials were conducted with Configuration 2, two of which included a paired untreated control. Each trial or pair of trials was conducted on a separate day. There was ≈30 min lag between the start of each trial when paired trials were conducted.

Statistical analysis.—The criterion we used to evaluate the effectiveness in the bioassays was recapture of significantly more ($P < 0.05$) ticks in treatments than controls. For instance, when natural populations were tested, we were interested in

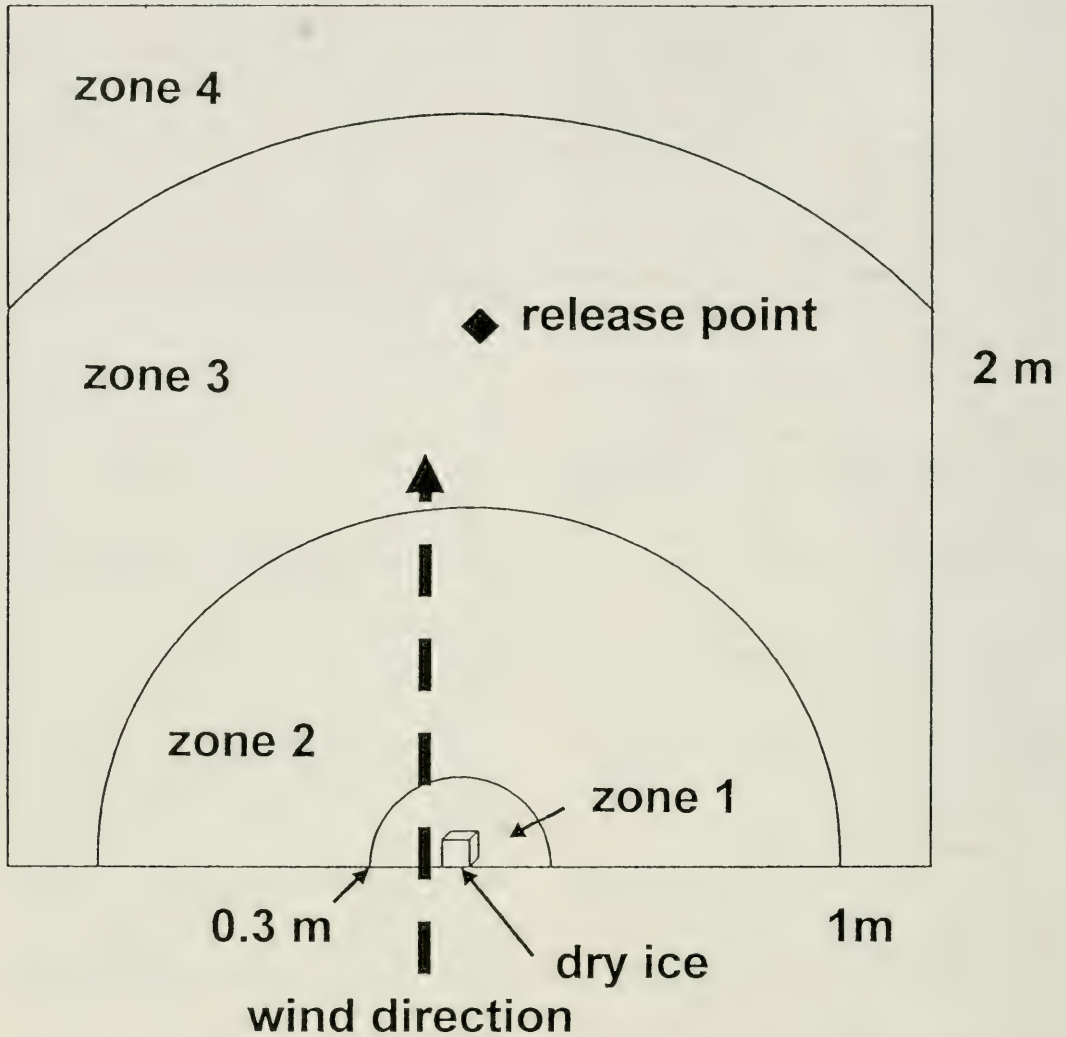


Fig. 2. Diagram showing sheet with bait location, recapture zones, tick release point for Configuration 2, and alignment with wind at start of bioassay. The sheet was edged with masking tape to confine released ticks and exclude ticks from natural population. Note that Configuration 2 allows for greater changes in wind direction without attracted ticks becoming stuck on masking tape than Configuration 1 with bait in a corner.

whether significantly more ticks were on dry ice sheets than untreated control sheets. When marked ticks were released on sheets, we focused on whether more ticks were recaptured in zones 1 and 2 on dry ice sheets than the corresponding zones on control sheets. The G statistic was used to determine significance between numbers of ticks in corresponding recapture zones on paired sheets (one baited with dry ice and the other an untreated control) for the release-recap-

ture trials, and between dry ice sheets and control sheets when natural tick populations were tested (Bishop et al. 1975).

RESULTS

Numbers of *A. americanum* nymphs from natural populations counted on the cloths with dry ice baits placed in forest habitats were highly variable. Even though the locations where the sheets were placed were determined by finding nymphs there

Table 1. Efficacies of combinations of the number of *A. americanum* nymphs released, their release point and recapture area are manifested in the proportion of trials in which statistical significance ($P < 0.05$) occurred comparing dry ice-baited and control sheets.

Comparison	Configuration/ experiment ^a	Distance (m) ticks released from bait ^b	No. ticks released in each trial	Recapture zone(s) ^c	No. significant trials/total ^d
1	1/1	1.01	10	1	1/4
2		2.01	10	1	2/4
3		1.01	10	1 + 2	1/4
4		2.01	10	1 + 2	4/4
5	1/2	1.01	15–19	1	2/4
6		2.01	15–19	1	2/4
7		1.01	15–19	1 + 2	4/4
8		2.01	15–19	1 + 2	4/4
9	2	1.50	50	1	1/2
10		1.50	50	1 + 2	2/2

^a Configurations depicted in Fig. 1 and 2; Experiments 1 and 2 each consisted of 4 trials.

^b Distances directly downwind from dry ice or control where nymphs were released.

^c Zone 1 (<0.3 m from bait), zone 2 (0.3–1.0 m from bait)

^d Number of trials in which significantly more nymphs were recaptured on dry ice-baited sheets than on control sheets over total number of trials.

during pre-trial flagging, and the trials lasted 2 h, four dry ice sheets had ≤ 2 ticks on them. As many as 69 and 71 nymphs were found on other dry ice sheets, with an overall average of 17.5 ± 8.7 nymphs. On sheets without dry ice, 0–3 ticks were found with an average of 0.9 ± 0.3 nymphs. For 6 of the 11 dry ice-control pairs, there were significantly more ($P < 0.05$) ticks on the dry ice sheet than the control sheet.

In Configuration 1 Experiment 1, when 10 *A. americanum* nymphs were released 1.01 and 2.01 m downwind of the dry ice bait or control, significantly greater numbers of ticks from the 1.01 m release were in Zone 1 (<0.3 m from bait) of the dry ice sheet than the control at 1 h after release in just one of four trials (Table 1). Of nymphs released at 2.01 m, significantly more ($P < 0.05$) were in the Zone 1 of the dry ice sheet than the control in two trials. Of the ticks released 1.01 m from the bait, significantly more ($P < 0.05$) were recaptured <1 m from the bait (Zones 1 and 2 combined) on the dry ice sheets than the control sheets in one of the trials. However, in all trials, of the 10 ticks released at 2.01 m, there were significantly more ($P < 0.05$) in Zones 1 and 2 combined of the dry ice

sheets than the controls. In the trials using 15–19 nymphs released at 1.01 and 2.01 m in Configuration 1 Experiment 2, there was a similar pattern (Table 1). Significantly greater ($P < 0.05$) attraction to dry ice than the control was observed in half the trials in which ticks were released at 1.01 and 2.01 m from the bait and recaptured in Zone 1. In all the trials, significantly greater numbers of ticks ($P < 0.05$) released at 1.01 and 2.01 m were recaptured in Zones 1 and 2 combined of the dry ice sheet compared to the control sheet. Temperatures ranged from 23.8–37.2°C and 23.3–30.0°C in Experiments 1 and 2 respectively; the RH varied from 32–57% and 34–61% in Experiments 1 and 2.

For Configuration 2, in which the dry ice was placed at the midpoint of one edge of the sheet and 50 nymphs were released 1.5 m downwind of it, an average of 12.6 ± 3.8 ticks (25.2%) was recaptured in Zone 1 in the five trials (two paired with an untreated control and three unpaired). An additional 28.4% of the nymphs, having moved ≥ 0.5 m toward the dry ice, were in Zone 2, whereas 1.6% were in Zone 4 (>2 m from the bait). Of the remaining ticks (44.8%) recaptured in Zone 3 (1–2 m from

the bait) in which they were released, some moved as far as the edge of the sheet (Fig. 2). Among the Configuration 2 dry ice trials, there was considerable variation in the numbers of ticks recaptured in Zone 1, which ranged from 1 to 20. In the two Configuration 2 trials using a simultaneous control sheet significantly more ($P < 0.05$) ticks were recaptured in Zones 1 and 2 combined on the dry ice sheets than the controls in both trials. There was no significant difference for Zone 1 in either trial. Both trials with simultaneous treatment and controls had the fewest ticks recaptured in Zone 1. During the Configuration 2 trials, temperature and RH varied less than in the Configuration 1 trials, 24.0–30.9°C and 48.5–62.7% RH respectively. Winds were light (≤ 10.5 km/h) and intermittent, with periods of calm. The direction of the winds often shifted and sometimes drastically (e.g., east to west). The individual dry ice blocks lost 170–273 g, i.e., 51.8–89.5% of their original weights. Levels of airborne CO₂ at the end of each trial at the release point (1.5 m from the dry ice) were indistinguishable from the background in all trials. At 1.0 m from the dry ice (in line with release point) the level of CO₂ was high (1350 ppm above background) in only one trial. At 0.3 m from the dry ice, levels were ≥ 450 ppm above background in all trials but one during which the wind changed direction 180° sending the CO₂ plume directly off the sheet.

DISCUSSION

The trials with paired dry ice and control sheets to attract ticks from natural populations showed that dry ice attracted significantly more *A. americanum* nymphs in 54.5% of the pairs. There was considerable variability in the numbers of nymphs from the natural population attracted to the dry ice baits. The principal disadvantage of testing the efficacy of an experimental attractant with natural populations is that the numbers of ticks available to be attracted is unknown. If ticks were distributed uniform-

ly in appropriate habitats, there could be some confidence that a field test of an experimental attractant against a natural population of ticks would yield meaningful data. However, these data (0–73 nymphs on dry ice sheets) showed a clumped distribution of *A. americanum* nymphs. No nymphs were captured at some locations even though ticks had been found by pre-trial flagging in the immediate area where the sheets were placed. Dragging/flagging for host-seeking ticks is thought to capture only a small portion (8–25%) of the ticks present in the area covered (Sonenshine et al. 1966, Carroll et al. 1991). A given number of ticks attracted could be a small portion of a dense population of available ticks or large portion of few available ticks. By releasing known numbers of host-seeking ticks on sheets or marked ticks on the leaf litter or substrate, one can ascertain the effectiveness and reliability of an experimental attractant with some confidence (Falco and Fish 1991).

In developing a behavioral bioassay to evaluate attractants, the dimensions of the arena and spatial relationships of the attractant bait and test organism are of particular concern. These trials suggest that using recaptured ticks in Zone 1 (< 0.3 m from the bait) as the standard for attraction is too exclusive. In the paired dry ice-control trials with Configurations 1 and 2, numbers of ticks in Zone 1 discriminated significance between dry ice and untreated controls in no more than half the trials. In contrast, Zones 1 and 2 (< 1.0 m from the bait) of the dry ice sheets had significantly more recaptured ticks that had been released at 1.5 and 2.01 m from the bait than that of the control sheets in all the trials. To reach zone 2 these ticks would have had to have advanced ≥ 0.5 m toward the bait within an hour, a good indication of attraction. Drastic shifts in wind direction or cessation of wind during a trial may make the < 0.3 m zone an unrealistic standard for assessing attraction.

It is desirable to minimize the number of

ticks released in the bioassays, because of time required to mark and recapture them. As seen in the results with Configuration 1, 10 ticks were too few to discriminate between dry ice and no treatment with much consistency. The instances of significance were observed in three of eight comparisons (four trials, two release points) for Zone 1 and five of eight comparisons for Zones 1 and 2 combined, when 10 ticks were released. However, when 15–19 ticks were released, significance was observed in four of eight comparisons for Zone 1 and all eight comparisons for Zones 1 and 2 combined. Other factors than numbers cannot be excluded as possible influences on the instances of significance. But even using 50 ticks was no guarantee of significance in all cases. In the two paired dry ice/control trials with Configuration 2, significance for zone 1 was achieved once.

These results show that variability of wind speed and direction greatly influences testing of air-borne attractants in the field. A constant unidirectional wind flow would be ideal for testing with ticks released downwind. For testing attractants with natural populations, changes in wind direction are preferable because more ticks distributed around a source are likely to be exposed to the shifting plume of attractant. None of the methods we used, including that with natural tick populations, was useful when winds ceased. With no wind there is little dispersion of the attractant, although gravity may allow CO₂ to flow down hill. At the conclusion of one Configuration 2 trial (dry ice on the side of the sheet), wind was undetectable with the wind meter. In that trial, only 1 tick (of 50 released) was found <0.3 m from the bait (zone 1), compared to an average of 12 ticks in the <0.3 m area when there were intermittent breezes of varying direction.

In tests with the CO₂ source located in the corner of the sheet, we sometimes observed that a cluster of ticks (e.g., 5–8) was stuck in the masking tape barrier on one edge of the sheet) 0.3–1 m from the bait.

These observations were coincident with shifts $\geq 45^\circ$ in the direction of the wind from the direction at the start of test (parallel to the diagonal of sheet). As the nymphs advanced up the gradient of the CO₂ plume, they followed the shifting plume as it passed over the edge of the sheet, and became trapped on the masking tape barrier. By locating the CO₂ source at the midpoint of one side, shifting winds could swing the plume almost 90° to either side of the starting direction without drawing advancing ticks into the masking tape. Nevertheless, the greater leeway for wind shifts obtained by placing the dry ice at the midpoint of the upwind side of the sheet did not accommodate for all conditions that occurred during the trials. A wind shift of $\geq 180^\circ$ (based on the direction of the marking flag and the visible CO₂ plume at the end of the trial) occurred during one Configuration 2 (dry ice at midpoint of sheet) trial and was associated with a cluster of nymphs caught in the tape in the 0.3–1 m zone. Nonetheless, it seems preferable to locate the bait source on a side of the sheet rather than in a corner.

Placing the dry ice in the center of a sheet and releasing ticks around the bait (in groups at the cardinal directions or evenly distributed), as might occur ideally in nature, would not necessarily improve the proportion of ticks that came to the bait. However, it would ensure that some ticks are exposed to the attractant if any breeze occurs. A very variable wind would expose the most ticks, but presumably they would take a tortuous route in the shifting CO₂ gradient to the bait, a route which would be longer than in a unidirectional wind and take more time. If ticks were evenly distributed in a circle around a bait, only about half would be exposed to the attractant if the wind direction changed as much as 180° (e.g., a shift from west to east). Ticks could also move before a shifting plume reached them. A smaller pool of ticks to attract from would increase the likelihood of equivocal results. For best results, using the methods

described in which ticks are released directly downwind of an attractant source, there should be a steady or intermittent breeze that shifts $<90^\circ$ from the direction at the onset of the trial.

Similar trials could be conducted using a 1 m² (or smaller) sheet and releasing known numbers of marked ticks on the natural ground cover. Ticks probably traverse a slightly undulating sheet more quickly than they can pass over and through the tangle and clutter of stems, broken twigs and curled leaves on the forest floor. Leaf litter and vegetation would reduce the range from which attracted ticks can reach the bait in a limited time. It is unclear from our tests to what degree, if any, marking the ticks with dusts affected their ability to respond to CO₂. However, sufficient numbers of ticks moved >0.5 m and 1.0 m into Zone 2 (0.3–1.0 m from the bait) to consistently show a significantly greater response than ticks on control sheets. Nymphs and adults of the blacklegged tick, *Ixodes scapularis* Say marked with dusts and released in a wooded habitat were recaptured days and weeks later ≥ 5 m from their release point (Carroll and Schmidtman 1996).

Exposing sufficient ticks to an attractant is critical to the success of the bioassay. By releasing known numbers of ticks under partially controlled field conditions, more reliable results can be obtained for attraction bioassays than by using natural tick populations. Based on the results of these trials, recommendations for evaluating a tick attractant under field conditions are: 1.) use Configuration 2 (allows for greater shifts in wind direction), 2.) release ≥ 15 ticks at a chosen release point, 3.) release ticks 1.5–2.01 m from bait, 4.) use ticks recaptured within a radius of 1 m from the bait as the basis for discriminating between treatments or treatment and control, and 5.) conduct trials when wind varies $<180^\circ$ and is not interrupted by prolonged periods of calm.

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